

## Supplementary Materials for

### Stepwise phosphorylation of leukotriene B<sub>4</sub> receptor 1 defines cellular responses to leukotriene B<sub>4</sub>

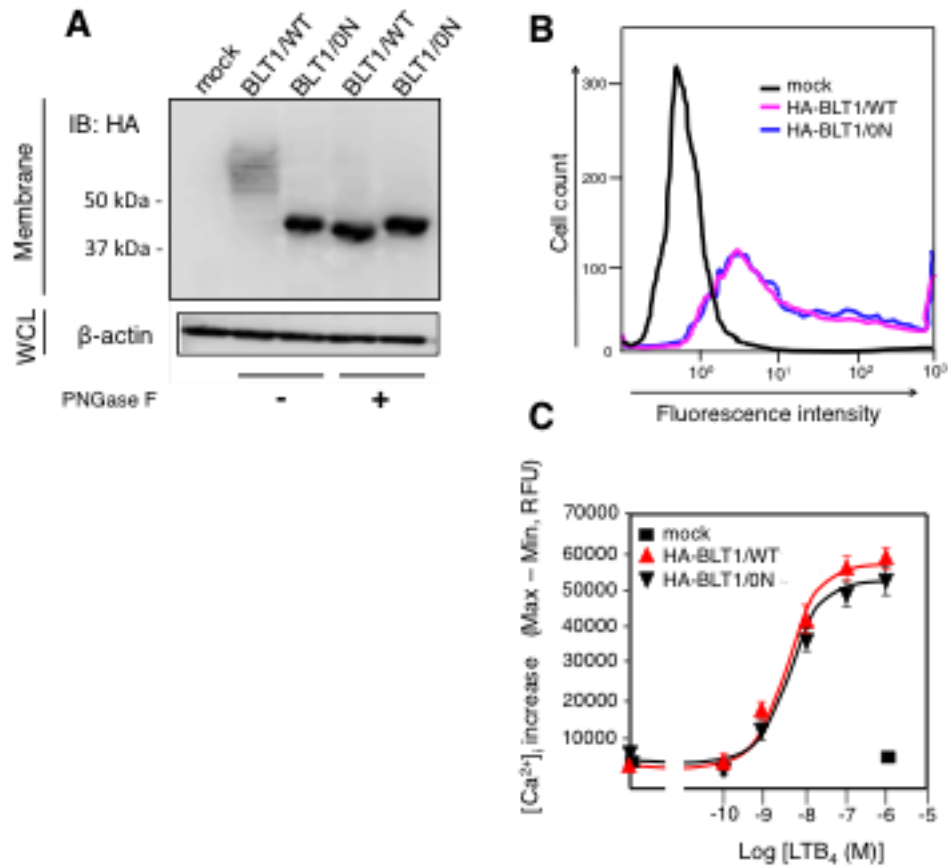
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**Fig. S1. Properties of N-glycosylation-deficient HA-BLT1/0N.**

HeLa cells were transfected with the indicated receptors. **(A)** Membrane fractions were treated with PNGase-F and subjected to SDS-PAGE. Receptors were detected by immunoblotting (IB) using an antibody recognizing the HA tag. **(B)** Cells were stained with the HA antibody followed by PE-conjugated secondary antibody, and HA-positive cells were sorted. **(C)** Intracellular Ca<sup>2+</sup> mobilizations elicited by LTB<sub>4</sub> were evaluated in HeLa cells expressing the indicated receptors. All data are representative of at least three independent experiments.

Human BLT1 MNTTSSAAPPSGLVGFISLLAIILLSVLAVGLPGNSFVVWSILKRMQRKSVTALMVLNLALAD

Mouse BLT1 MAANTTSSAAPSPGGMSISLLPIVLLSVLAVGLPGNSFVVWSILKRMQRKSVTALLVNLALAD

Rat BLT1 MAANTTSSAAPSPGGMSISLLPIVLLSVLAVGLPGNSFVVWSILKRMQRKSVTALLVNLALAD

Guinea pig BLT1 MDRNTTTTAAASPSGSNTFPIPLAMILLSVSMVVGLPNGTFFVWSILKRMQRKSVTALMVLNLALAD

Zebrafish BLT1 MATPLTPVFGSPVSAVVPAPSTPPSLPLSHQIGIAILVIAFVFGFPGNLFVVWSVLCVRVRRRSVTCLLILNLAVAF

TM3

Human BLT1 LAVLLTAPFFHLFLAQGT-WSFGLAGCRLCHYVCGVSMYASVLLITAMSLDRSLAVARPFVSKQLRKAMARRVLAGI

Mouse BLT1 LAVLLTAPFFHLFLARGT-WSFREMGRCLCHYVCGVSMYASVLLITIMSLDRSLAVARPFMSQKVRKTAKAFRWVLAGI

Rat BLT1 LAVLLTAPFFHLFLARGT-WSFEVTCGRCLCHYVCGVSMYASVLLITIMSLDRSLAVARPFVSKQVRKTAKAFRWVLAGI

Guinea pig BLT1 LAVLLTAPFFHLFLTWT-WSFKLAGCRLCHYICGVSMYASVLLITAMSLDRSLAVASPFMSQKVRKTAKARWLLVGI

Zebrafish BLT1 ALVLLSSPLFIRYLVGGKGWEPVCKTVHLYLCVMNMYASVLLICVMSMDRWLAVTKPFLSQRLRTAKRLLSIMLAI

TM4

Human BLT1 WVLSFLLATPVLAYRTVVPWKTNMS---LCFPYPYSEG-HRAFHLIFEAVTGFLPLFLAVVASVSDIGRRLQARRFRFS

Mouse BLT1 WVVSFLLAIPVLVYRTVK---WNNTL-ICAPNYENKE-HKVPHLLFEAITGFLPLFLAVVASVSDIGRRLQARRFRFS

Rat BLT1 WVVSFLLAIPVLVYRTVT---PKNRTL-ICDSRYPSDG-HKVPHLLFEAITGFLPLFLAVVASVSDIGRRLQARRFRFS

Guinea pig BLT1 WGSFLLATPVLAFRKVVK-LTNETD-LCLAVYPSDG-HKAFHLLFEAITGFPVPLFVVASVADISIRLVRVRFRHR

Zebrafish BLT1 WVMAFMLALPMFIFYRSVVQYKGPVIYLCNPNHWQSESHEIFQYLLSETLLGFLPLFAFIFLCYISVILRLRNAMFQRK

TM5

Human BLT1

Mouse BLT1

Rat BLT1

Guinea pig BLT1

Zebrafish BLT1

TM6

Human BLT1 RTGRLVVLIIILTAFAFWLPHYHVNLAEAGRALAGQAAGLGLVGKRLSLARNVLIALAFSSSSVNPVLYACAGGGL---

Mouse BLT1 RTGRLVVLIIILAFAAFVLPYHLVNLVEAGRTVAGWDKNS-PAGQRLRLARYVLIALAFSSSSVNPVLYACAGGGL---

Rat BLT1 RTGRLVVLIIILAFAAFVLPYHLVNLVEAGRTLAGWDKNS-PAGQRLKLARYVLIALAFSSSSVNPVLYACAGGGL---

Guinea pig BLT1 RTGRLVVIILAFAAFVLPYHVVDLVEGSRVLAGTLQDS---KQQLRNARKVCIALAFSSSSVNPVLYACAGGGL---

Zebrafish BLT1 GRGNFLILIIIIQVIGVGMQGSSSFNLNAVKG-----RPNVTAFAFMSSSSVNPAAFTLFWLPHYHL

TM7

Human BLT1

Mouse BLT1

Rat BLT1

Guinea pig BLT1

Zebrafish BLT1

Helix8

Human BLT1 -----LRSGVGVFVKLLLEGTSSEASTRRGSLGQTPARSGPAALEPGPSESITASSPFKLNELN

Mouse BLT1 -----LRSGVGVFVKLLLEGTSSEVSSSTRRGGLTVQTPKDTACPEPEPGPDSFMTSSSTIPESK

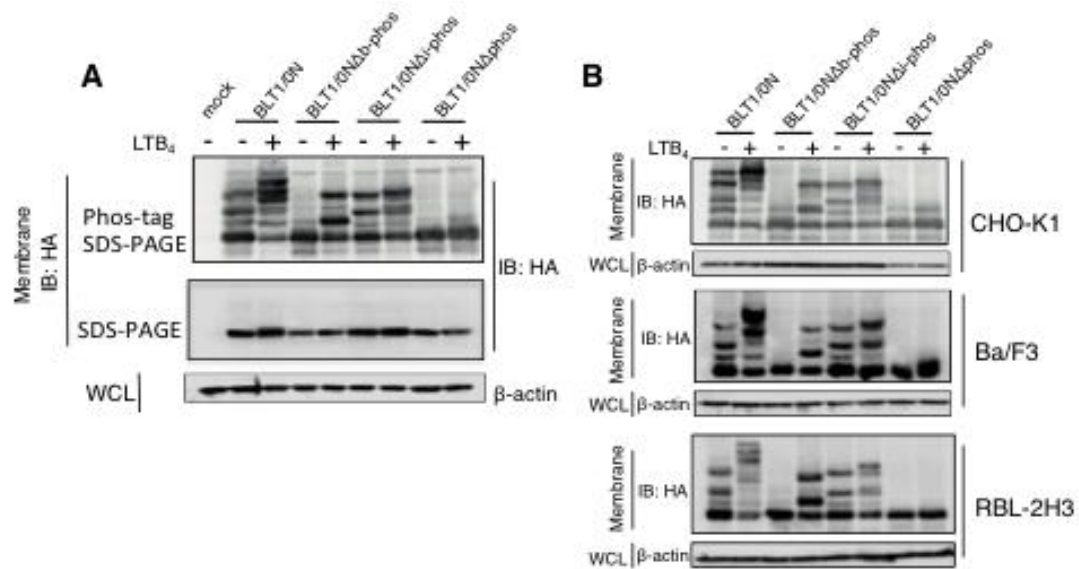
Rat BLT1 -----LRSGVGVFVKLLLEGTSSEVSSSTRRGGLTVQTPKATPTCEPEPGPDSGSMSTSSSTIPESK

Guinea pig BLT1 -----LRSGVGVFVKLLLEGTSSEAFSTRRGGLTAQTVKGIPTAPEPGPDSGSLDLGQSESD

Zebrafish BLT1 INVLYVFAGSSSHIRQAGLGFMAKLFEGTNSEMGSSRSRSTRSSRGSSNTENSVFTKLSVKLNKRGDGAETHDGEETLA

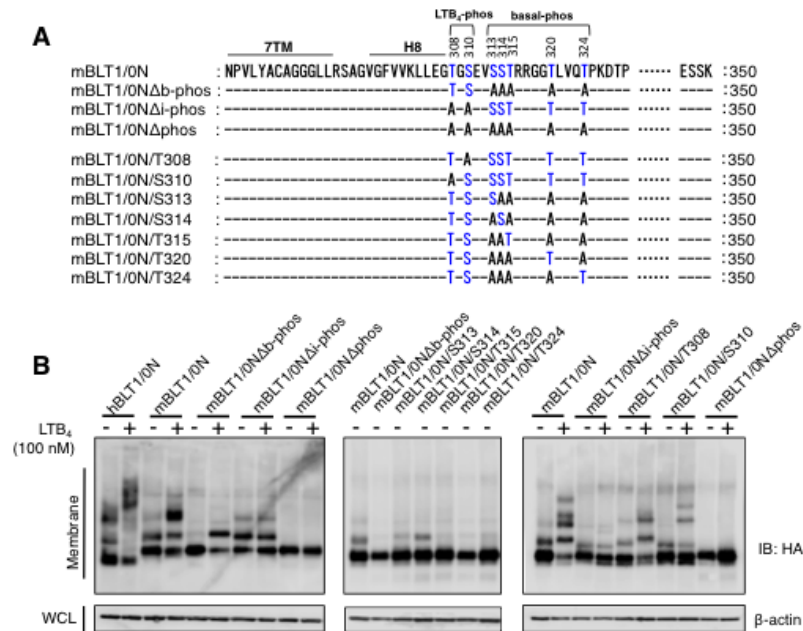
**Fig. S2. Conserved Ser and Thr residues in the cytoplasmic domains of human, mouse, rat, guinea pig, and zebrafish BLT1.**

Putative transmembrane domains and helix 8 regions are labeled as TM1-7 and Helix-8, respectively. Highly conserved Ser and Thr residues in the intracellular domains are indicated in blue. The region containing 7 phosphorylation sites in human BLT1 is boxed.



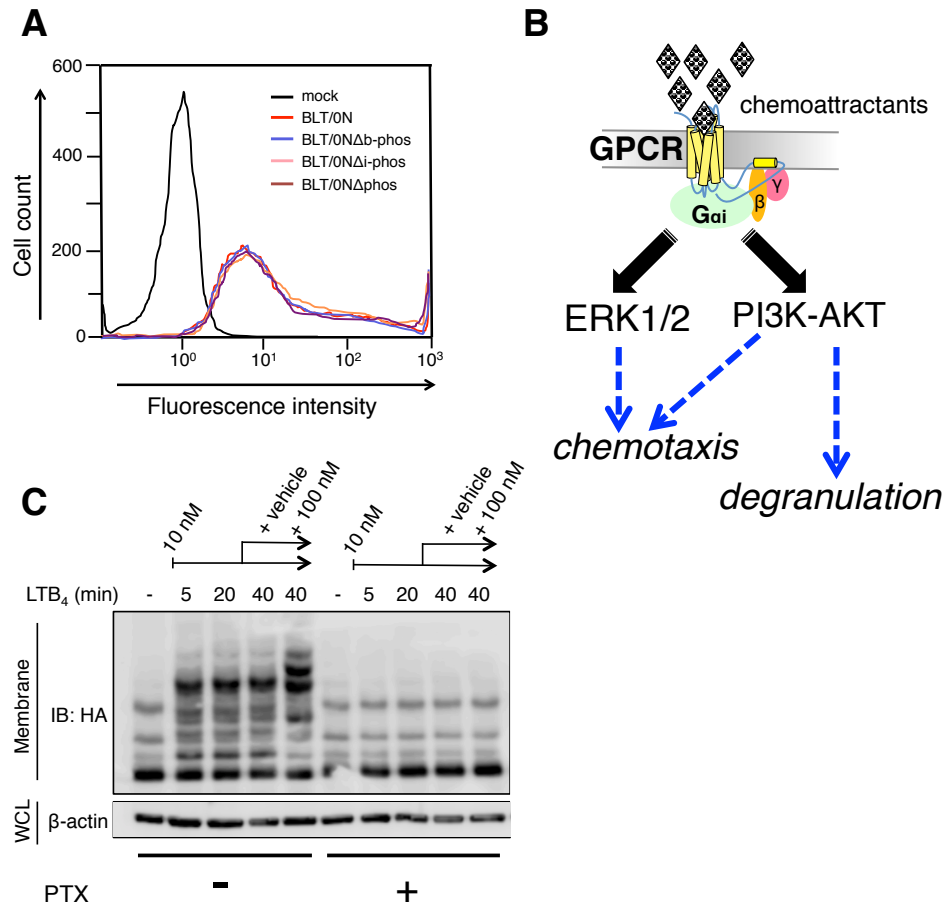
**Fig. S3. Confirmation of residues essential for HA-BLT1/0N phosphorylation.**

The human BLT1 mutants deficient in the basal ( $\Delta b$ -phos), LTB<sub>4</sub>-induced ( $\Delta i$ -phos), or both basal and LTB<sub>4</sub>-induced ( $\Delta$ phos) phosphorylations were constructed as indicated in Fig. 2D. **(A)** HeLa cells expressing the indicated receptors were stimulated with 100 nM LTB<sub>4</sub> for 40 min. Membrane fractions were subjected to Phos-tag SDS-PAGE and SDS-PAGE. Receptors were detected by immunoblotting (IB) for HA tag. **(B)** Similar experiments as in (A) were carried out in CHO-K1, Ba/F3, and RBL-2H3 cells. All data are representative of at least three independent experiments.



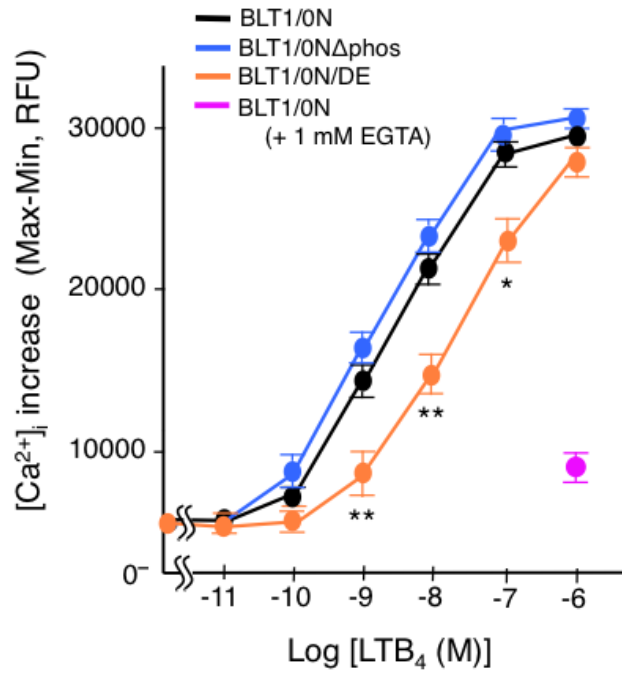
**Fig. S4. Phosphorylation of mouse BLT1.**

(A) The indicated mutants of HA-tagged mouse BLT1 were constructed to determine the phosphorylated residues. Ser and Thr residues conserved between human and mouse BLT1 are shown in blue. (B) HeLa cells expressing these mutants were stimulated with or without 100 nM LTB<sub>4</sub> for 20 min. The membrane fractions were subjected to Phos-tag SDS-PAGE and immunoblotting for HA. All data are representative of at least three independent experiments.



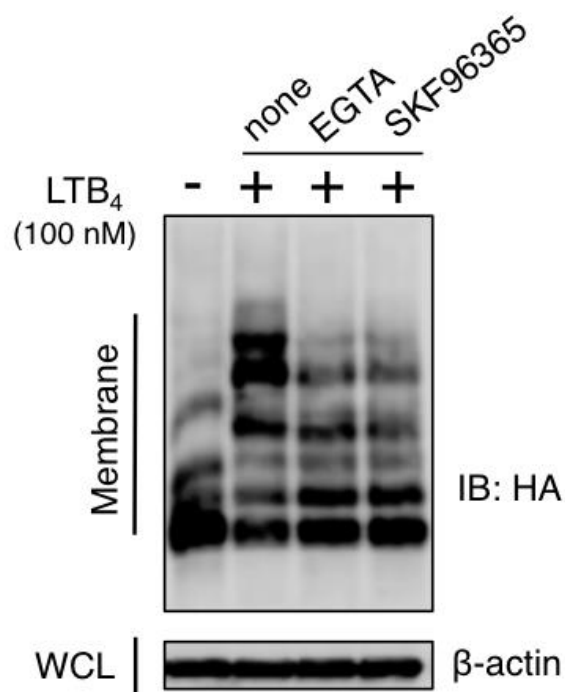
**Fig. S5. Phosphorylation at LTB<sub>4</sub>-induced and basal sites through G<sub>i</sub>.**

(A) HeLa cells expressing the indicated HA-tagged receptors were stained with the antibody recognizing HA followed by the PE-conjugated secondary antibody, and HA-positive cells were sorted. (B) Schematic model of the signaling pathways operating through GPCRs activated by chemoattractants. Although the stimulation of G<sub>i</sub> is a prerequisite, activation of both the ERK1/2 and PI3K-AKT pathways is required for chemotaxis, whereas activation of only the PI3K-AKT pathway is necessary for degranulation. (C) HeLa cells expressing HA-BLT1/0N were stimulated as indicated. For PTX treatment, cells were preincubated with 100 ng/ml PTX overnight. Membrane fractions of these cells were subjected to Phos-tag SDS-PAGE and immunoblotting (IB) for HA. All data are representative of at least three independent experiments.



**Fig. S6. LTB<sub>4</sub> dose dependency of intracellular [Ca<sup>2+</sup>] increase.**

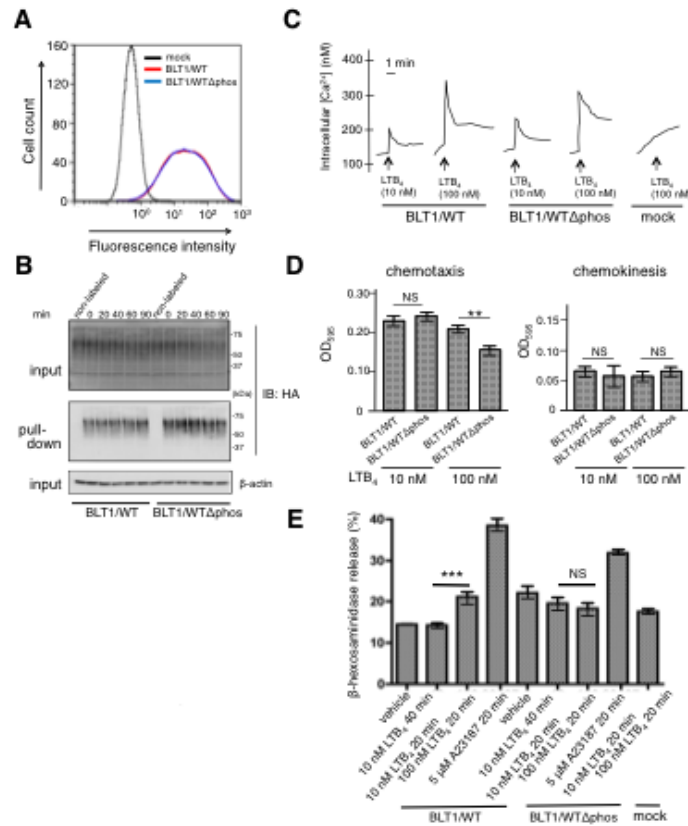
Intracellular Ca<sup>2+</sup> mobilizations elicited by LTB<sub>4</sub> were evaluated in CHO-K1 cells expressing the indicated receptors. For EGTA treatment, the medium was replaced with Ca<sup>2+</sup>-free medium containing 1 mM EGTA prior to LTB<sub>4</sub> application. Data are means  $\pm$  SEM,  $n = 3$  experiments. \* $P < 0.05$ , \*\* $P < 0.01$  by two-way ANOVA followed by Tukey's *post hoc* test. All data are representative of at least three independent experiments.



**Fig. S7. Effect of blockage of the  $[Ca^{2+}]$  increase on BLT1 phosphorylation.**

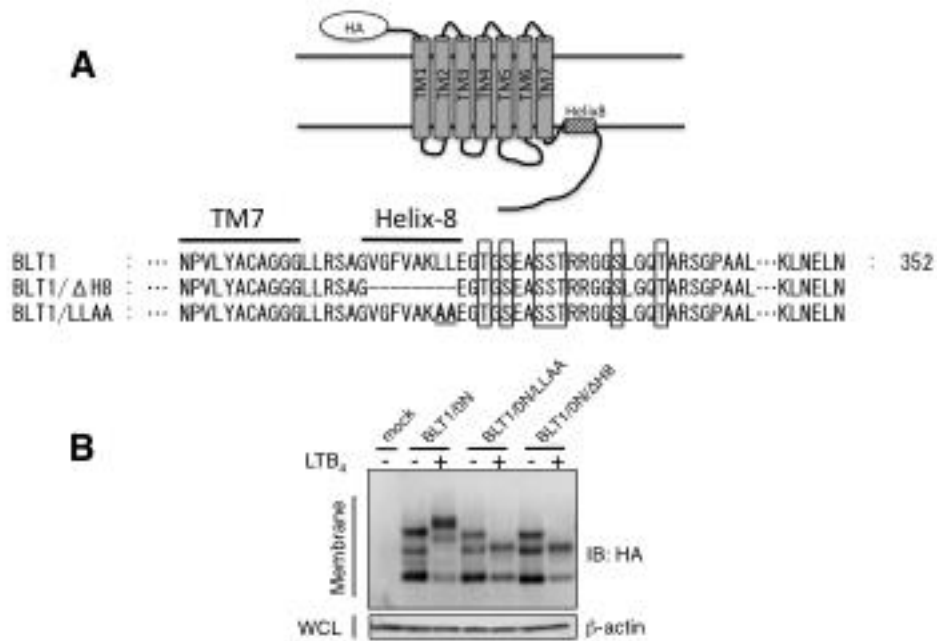
HeLa cells expressing HA-BLT1/0N were stimulated with 100 nM LTB<sub>4</sub> for 20 min. For EGTA treatment, the medium was replaced with Ca<sup>2+</sup>-free medium containing 1 mM EGTA prior to 100 nM LTB<sub>4</sub> application. For SKF96365 treatment, cells were preincubated with 100 μM SKF96365 for 10 min before LTB<sub>4</sub> application. Membrane fractions were subjected to Phos-tag SDS-PAGE and immunoblotting for HA. All data are representative of three independent experiments.





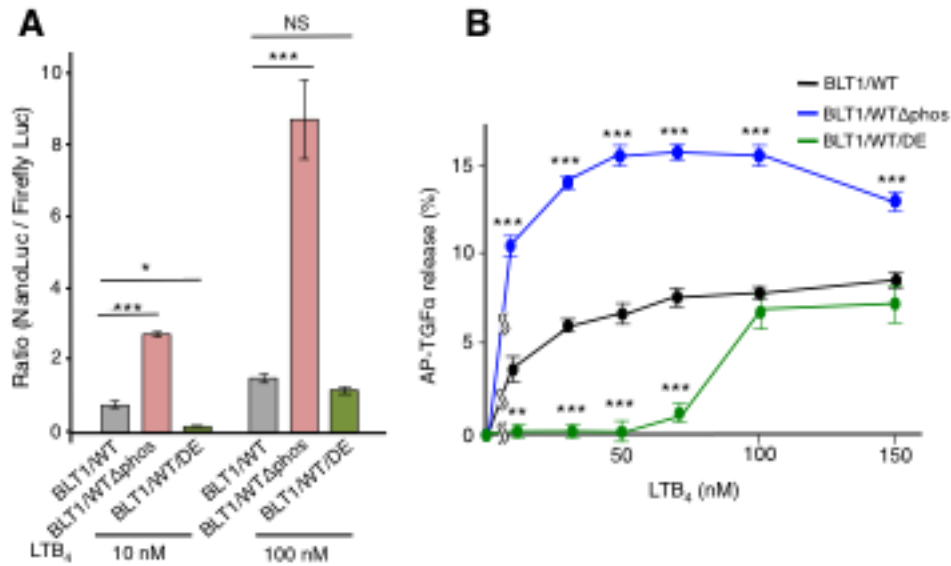
**Fig. S8. Effect of phosphorylation deficiency on the functions of wild-type BLT1.**

(A) HeLa cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos were stained for HA, and HA-positive cells were sorted. (B) Biotinylated HA-BLT1/WT or HA-BLT1/WTΔphos on the surface of HeLa cells was stimulated with 10 nM LTB<sub>4</sub> for the indicated time. The biotinylated BLT1 molecules were collected by Streptavidin pull-down. Receptors were detected by immunoblotting for HA. (C) LTB<sub>4</sub>-induced intracellular Ca<sup>2+</sup> mobilizations were examined in CHO-K1 cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos. (D) Chemotaxis and chemokinesis elicited by 10 nM and 100 nM LTB<sub>4</sub> were evaluated in CHO-K1 cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos. Data are means ± SEM, *n* = 5 experiments. NS, not significant; \*\**P* < 0.01 by two-way ANOVA followed by Tukey's *post hoc* test. (E) Release of β-hexosaminidase in RBL-2H3 cells expressing the indicated receptors was quantified. Column 1, vehicle; column 2, 10 nM LTB<sub>4</sub> for 40 min; column 3, 10 nM LTB<sub>4</sub> for 20 min then addition of 100 nM LTB<sub>4</sub> for 20 min; column 4, A23187 for 20 min. Data are means ± SEM, *n* = 3 experiments. NS, not significant; \*\*\**P* < 0.001 by one-way ANOVA followed by Tukey's *post hoc* test. All data are representative of at least three independent experiments.



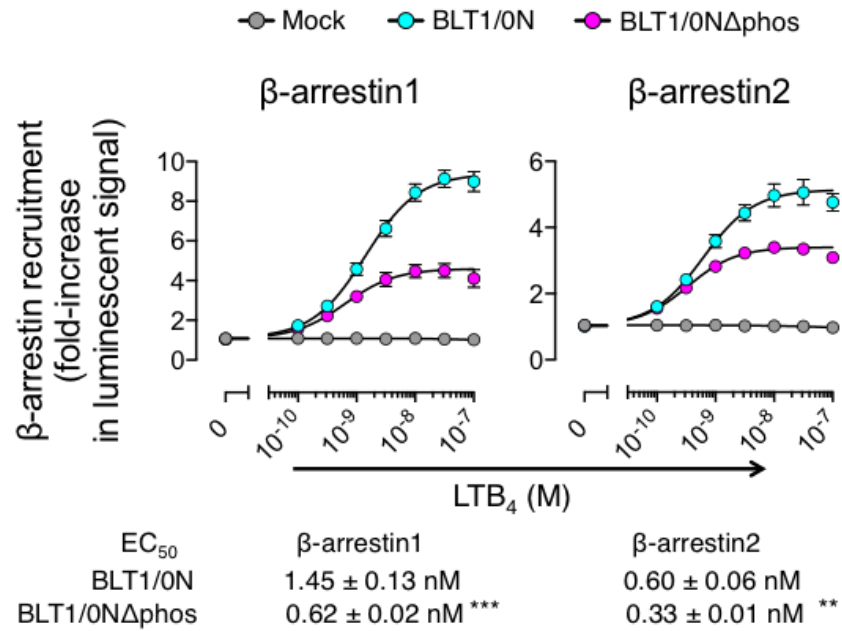
**Fig. S9. Effect of helix 8 disruption on BLT1 phosphorylation.**

(A) Two HA-BLT1/ON mutants were constructed to examine the importance of helix 8 in the phosphorylation of BLT1 (25). Putative transmembrane domain-7 and helix 8 are labeled as TM7 and Helix-8, respectively. The 5 basal phosphorylation and 2 LTB<sub>4</sub>-induced phosphorylation sites are boxed. (B) HeLa cells expressing the indicated receptors were treated with 100 nM LTB<sub>4</sub> for 40 min. Membrane fractions were subjected to Phos-tag SDS-PAGE. Receptors were detected by Western blotting for HA. All data are representative of at least three independent experiments.



**Fig. S10. Importance of phosphorylation for the ligand sensitivity of BLT1.**

(A) HEK293T cells expressing SRE-NanoLuc, PGK-firefly luciferase and the indicated receptors were treated with 10 nM or 100 nM LTB<sub>4</sub>. The ratios of NanoLuc to firefly luciferase activity are shown. Data are means  $\pm$  SEM,  $n = 3$  experiments. NS, not significant; \* $P < 0.05$ , \*\*\* $P < 0.001$  by one-way ANOVA followed by Tukey's *post hoc* test. (B) HEK293T cells expressing AP-TGF $\alpha$ , G $\alpha_{q/i1}$  and the indicated receptors were stimulated under various concentrations of LTB<sub>4</sub>, and the AP-TGF $\alpha$  release into the medium was determined. Data are means  $\pm$  SEM,  $n = 3$  experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by two-way ANOVA followed by Tukey's *post hoc* test.



**Fig. S11. Effect of phosphorylation on β-arrestin binding to BLT1.**

LTB<sub>4</sub>-induced β-arrestin recruitment to BLT1 was assessed by using a NanoBiT system. Luminescent signals in HEK293 cells expressing LgBiT-βarr1 or LgBiT-βarr2 together with BLT1/0N or BLT1/0NΔPhos were measured before and after stimulation with LTB<sub>4</sub>. β-arrestin recruitment was expressed as fold-change luminescence signals. Data are means ± SEM. *n* = 4 experiments. \*\**P* < 0.01, \*\*\**P* < 0.001 by Student's *t*-test.

**Table S1. Primer sequences used to generate mutant BLT1s.**

Mutant	Forward primer	Reverse primer
1N(N2A)	gactacgccgccaactacatcttctg	cagaagatgtagtggcggcgtagtc
1N(N164A)	ccctggaaaacggccatgagcctgtgc	gcacaggctcatggccgtttccaggg
iL-1	cagaagcgcgctgtcgtgccctg	cagggcagcgacagcgcgttctg
iL-2A	ctagaccgcgcactggcggtg	caccgccagtgcgcggtctag
iL-2B	ctttgtggcccagaagctacgcgccaaggcg	cgccttggcgcgtagcttctgggccacaaag
iL-3A	gctgtggtggccgcctacgcggacataggg	ccctatgtccgcgtagggcgccaccacagc
iL-3B	cttcgccgcgcccgcgcggccgcgc	gcggccggcgcgccggcgcgccggaag
CT-1	ctgctgcgcgcccggcggtgggc	gcccacgcccgcgcgcgagcag
CT-2	ctggagggcgcgggcgccgagggctcc	ggacgcctcggcgcccgcgcctccag
CT-3	ccgagggcgccgcgcgcgcggcg	cccgcggcgcgcgccggcgccctcgg
CT-4	ggggggcgccctgggccagggcgtagg	cctagcggcctggcccagggcgcccc
CT-5	cccggccctgccgagggcctcactgcc	ggcagtgagggcctcggcagggcgggg
0N/S313	ccgagggcgccgcgcgcgcggcg	cccgcggcgcgcgccggagcctcgg
0N/S314	ccgagggcgccagcgcgcg	gcgcgcgctggccgcctcgg
0N/T315	ccgagggcgccgccagcgccgcggg	cccgcggcgcgctggcgccgcctcgg
0N/S320	ggggggcagcctgggccagggcgtagg	cctagcggcctggcccagggcgcccc
0N/T324	ggggggcgccctgggccagaccgtagg	cctagcgggtcggcccagggcgcccc
$\Delta$ b-phos	ctgggccagggcgtaggagc	gctcctagcggcctggcccag
0N/S310	ctggagggcgcgggctccgagggctcc	ggacgcctcggagcccgcgcctccag
0N/T308	ctggagggcacggcgccgagggctcc	ggacgcctcggcgcccgtgcccctccag
$\Delta$ i-phos	ctggagggcgcgggctccgagggctcc	ggacgcctcggagcccgcgcctccag
$\Delta$ phos	ctggagggcgcgggcgccgagggcgcc	ggccgcctcggcgcccgcgcctccag
pMK-HA	gtgccagactacgcatggccaactacatcttct	gagggcgggatccta
BLT1/0N	ggcgtagtctggcacgt	taggatccgcccctctc
mBLT	atggctgcagccaactacatctcctgcagc	tcacttcgaagactcaggaatggtgg
mBLT/0N	cagtaaaatggaacgccaggactctgatc	gatcagagtcctggcggtccatttactg
mBLT/ $\Delta$ b-phos	ctggagggcactggctcggaggtggccgcccgc cgccgccccggcgctctgtgtccagggcccgaag gac	gtccttcggggcctggaccagagcgcccccgcg gcggcgggggccacctccgagccagtgcctt ccag

mBLT/ $\Delta$ i-phos	ctggagggcgctggcgcgaggtgtccagcacc cgccgcgggggcactctggtccagacccgaag gac	gtccttcgggggtctggaccagagtgtccccgcg gcgggtgctggacacctccgcgccagcgcctc cag
mBLT/ $\Delta$ phos	ctggagggcgctggcgcgaggtggccgccgcc cgccgcgggggcgctctggtccagggccgaag gac	gtccttcggggcctggaccagagcgtccccgcg gcgggcggcgggccacctccgcgccagcgcct ccag
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